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Research Paper

Pre-analytical factors related to the stability of ethanol concentration during storage of ante-mortem blood alcohol specimens

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ABSTRACT

Sterile ante-mortem blood specimens were spiked with ethanol at the South African blood alcohol legal concentration limits of 0.20 g/L and 0.50 g/L and were stored in tubes containing sodium fluoride over a period of twenty-nine weeks under refrigeration (4 °C) and at room temperature (22 °C) to study the stability of the ethanol concentrations over time. Those stored under refrigeration were found to be stable, while a significant decrease in ethanol concentration at 99% confidence was observed in those stored at room temperature.

Additional blood specimens, also spiked with ethanol, were inoculated with the dimorphic fungus *Candida albicans* at five different levels (1×10^6 cells/mL, 5×10^5 cells/mL, 1×10^4 cells/mL, 5×10^3 cells/mL and 5×10^1 cells/mL) and stored with and without sodium fluoride at 4 °C and 22 °C. The ethanol concentrations were monitored for nine weeks unless no fungal colonies were detected. Regardless of the presence or absence of NaF in samples – sterile or otherwise – storing specimens at 4 °C was sufficient to maintain the integrity of blood alcohol concentrations.

The ethanol analyses were performed with an in-house validated isotope dilution gas chromatography-mass spectrometry analytical method on newly opened specimens once a week after which significance testing was performed to draw conclusions regarding changes in ethanol concentrations with measurement uncertainty in mind.

1. Introduction

Blood alcohol analysis is one of the most often requested forensic tests in South Africa and is mostly used for law enforcement purposes. The legal limits in South Africa are 0.20 g/L and 0.50 g/L for professional drivers and public drivers respectively.[1] The standard protocol requires that a qualified phlebotomist draws blood from an antecubital vein of a driver within 2 h after arrest. Blood is collected directly into a sterile evacuated tube containing potassium oxalate as anticoagulant and sodium fluoride (NaF) as inhibitor.[2,3]

Protocol prescribes that the specimens be kept cool for the period of transportation to the laboratory, and thereafter be stored under refrigeration at 4 °C until analysis.[4] The sodium fluoride concentration should be above 1% after filling the tube with venous blood, and this concentration is routinely determined and reported as part of the blood alcohol test report.[3]

The reliability of blood alcohol test results is being questioned by defence teams due to claims in literature that the alcohol concentration may increase with time, as reported by Chang et al. and Blume et al. on post-mortem blood.[5,6] The relevance of studies making use of post-mortem blood attempting to explain changes in ante-mortem blood ethanol concentrations is questionable. In the presented study, ante-mortem blood was used. Yajima et al.[7] showed that the presence of *C. albicans* caused an increase in ethanol concentration, provided glucose was added, and that the ethanol production was proportional to the glucose concentration. It should be noted that no ethanol increase was observed in specimens that did not have added glucose.

Little emphasis however is placed on the fact that the majority of studies found in literature make use of either blood obtained from a blood bank, containing dextrose,[5] an additional substrate for microbial growth, or post-mortem blood, where the blood biochemistry has changed.[8] Neither of these adequately simulates the ante-mortem blood specimens routinely obtained for blood alcohol analysis. Nevertheless, the reasons for the changes in alcohol concentration are cited in literature as microbial contamination of blood specimens[5,6,9] and non-enzymatic oxidation.[10–12] Possible microbial contaminants capable of

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producing ethanol include *Candida albicans*, *Proteus* sp., *Escherichia coli*, *Staphylococcus* sp.[13] However, it has been shown that contamination with micro-organisms from post-mortem specimens introduced into ante-mortem specimens by diluters or pipettes can also cause a substantial decrease in blood alcohol concentration of the ante-mortem specimen.[4]

The stability of blood alcohol concentrations in blood specimens is of paramount importance. Should the ethanol concentration decrease it would only be to the benefit of the defendant and justice may not be served, while if it were to increase they might be prosecuted unfairly. It is thus imperative for a reliable and accurate blood alcohol result that the ethanol concentration remain unchanged from sampling to analysis – that is, with no *significant* increases or decreases.[14,15]

Fluoride acts as a potent inhibitor of enolase, one of the enzymes in the glycolysis pathway, whereby a micro-organism like *Candida albicans* can convert blood glucose into ethanol anaerobically.[16] It should be noted, however, that according to literature the absence of preservative (NaF) in contaminated blood specimens causes the whole blood glucose level to decrease more rapidly, thereby removing the major substrate of ethanolic fermentation.[17]

It should also be considered that ethanol is a volatile substance. As such it is possible that evaporation may cause a decrease in ethanol concentration.

In order to ensure the validity of test results, testing laboratories require procedures for safe handling, transport and storage of samples and reference materials in order to protect specimen integrity. This inherently necessitates the laboratory to prove analyte stability over the period of storage. Where there is a backlog of specimens in a laboratory this becomes even more critical. Pre-analytical studies simulating the storage conditions must therefore be conducted to assess the impact of storage on the final result.

In this study, an in-house validated gas chromatography – mass spectrometry isotope dilution analytical procedure was used to obtain information on the stability of ethanol concentration in ante-mortem blood specimens inoculated at different concentrations of *C. albicans*. In addition to being stored at either room temperature or under refrigeration, some specimens contained sodium fluoride (NaF) while others did not. The expanded measurement uncertainty of the analytical method was used to gauge the significance of any changes in blood alcohol concentration.

2. Materials and methods

2.1. Reagents and materials

A certified ethanol reference standard (200 g/L) was obtained from the National Metrology Institute of South Africa (NMISA) and stable isotope labelled ethanol-D6 (99%) was obtained from Sigma Aldrich, Midrand, South Africa.

Sodium hydrogen carbonate (99%) and sodium carbonate (99%) were purchased from Merck, Steinheim, Germany; pentafluorobenzoyl chloride (99%) (PFBCl) was obtained from Sigma-Aldrich, Midrand, South Africa; sodium hydroxide pellets (97.0%) were acquired from Merck, Worli, Mumbai.

All solvents were analytical grade and were used without further preparation.

Dichloromethane (pesticide grade) and isopropanol (99.9%) were obtained from Sigma-Aldrich, Steinheim Germany. Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich, Midrand, South Africa, while deionised water was sourced from Merck, Modderfontein, South Africa.

A *Candida albicans* ATCC 90028 strain was obtained from the Department of Medical Microbiology, Tshwane Academic Division (TAD), National Health Laboratory Service (NHLS). Phosphate Buffered Saline (PBS) (pH 7.2) was obtained from Thermo Fischer Scientific, Waltham, MA, United States of America. Agar plates containing Chloramphenicol ("C-plates") were prepared by Department of Medical Microbiology, TAD, NHLS. A 0.5 McFarland turbidity standard corresponding to 1.5×10^8 cells/mL was used to prepare a C. *albicans* suspension to be used as a stock solution.

2.2. Analytical method

An in-house validated GC-MS isotope dilution blood alcohol analytical method was employed. This involved an *in-situ* esterification of ethanol to form ethyl pentafluorobenzoate, followed by liquid-liquid extraction, and selected ion detection and quantitation against a stable isotope internal standard.

Briefly the procedure was as follows:

The specimen/standard solution (500 µL) was spiked with aqueous internal standard ethanol-D6 (50 µL, 1.164 g/L) and deproteinated with acetonitrile (700 µL). Saturated sodium bicarbonate solution (1000 µL) was added to the supernatant in a clean reaction tube. Pentafluorobenzoyl chloride (5% v/v, 1000 µL) in dichloromethane solvent was added and the mixture was shaken. The organic phase was transferred to a new reaction vessel and after being washed with saturated sodium bicarbonate solution (1000 µL), was dried completely under compressed air, reconstituted with dichloromethane and transferred into a conical insert in a 2-mL GC vial.

The validation figures of merit were as follows.

2.2.1. Linearity

Five non-weighted linear calibration graphs not forced through zero of relative response versus concentration including the above seven concentrations and reagent blank obtained on five separate days yielded an average correlation coefficient of $r^2 = 0.9945 \pm 0.002351$. The 95% confidence intervals for the gradient and intercept were 0.70607 ± 0.00935 and -0.00107 ± 0.03444 respectively. The residuals were examined and it was found that the data was homoscedastic over the calibration range.

2.2.2. Limits of detection and quantitation

A signal for ethanol could no longer be detected at the lowest spiked concentration of 0.000078125 g/L; however, a S/N ratio of 3:1 was obtained at 0.0125 g/L ethanol, and a S/N ratio of 10:1 at 0.025 g/L.

2.2.3. Bias

A non-weighted linear regression bias correction plot was obtained by plotting the average experimental concentrations of the three internal quality control levels (Y) of 0.215 g/L, 0.511 g/L, and 2.951 g/L versus the theoretical concentrations (X) of 0.2 g/L, 0.5 g/L and 3.0 g/L, and had the form $Y = BX + A$. The purpose of this correction plot is to correct for the bias at all concentrations in the linear range of the method, and not only at the discrete concentration levels. The actual bias at each of the three discrete concentrations above was 7.5%, 2.2% and −1.65% respectively. The 95% confidence intervals of the intercept and gradient were determined to be $A = 0.021 \pm 0.024$ and $B = 0.976 \pm 0.014$ respectively. It was thus determined that the method exhibited no additive bias, while it did display multiplicative bias - that is, a bias that is dependent on concentration.

2.2.4. Precision

Precision studies were performed by repeated measurements at the three internal quality control concentration levels of 0.20, 0.50 and 3.00 g/L ethanol in blood. The within batch precisions were found to be 6.5%, 3.8% and 5.5%, and the between batch precisions found to be 11.7%, 9.6% and 9.2% for the three internal quality control levels respectively (n = 20).

2.2.5. Selectivity

The principle of "identification" before "quantification" was applied and ion abundance ratios were used for identification of the ethyl pentafluorobenzoate. The 95% confidence intervals for abundance ratios were 0.250 ± 0.006 for 240/212; 0.208 ± 0.009 for 240/167 and 0.836 ± 0.036 for 212/167. If the ion ratios did not comply with these tolerances, corrective action was applied and the specimen submitted for reanalysis.

Analysis of blank matrix pooled blood obtained from 34 individuals did not indicate any signal at the specific retention time of the ethanol derivative (2.08 ± 0.12 min).

In addition, selectivity investigations were performed by spiking blank matrix pooled blood with isopropanol, acetaldehyde, methanol, acetone and propanol at a concentration level of 0.5 g/L. None of these were found to interfere with the signal for the ethanol derivative or the labelled ethanol derivative.

2.3. Instrumental conditions

An Agilent 7890 A GC system fitted with an Agilent 7683 Autoinjector and a 5975C mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) was used for mass spectrometric analysis. A mid-polar fused silica column (ZB5-MSi, 15 m × 0.25 mm, d_f = 0.25 μm, Phenomenex, California, USA) was used with sample injection (2 μL) in split mode (20:1). The inlet temperature was set at 250 °C and helium carrier gas at a constant flow rate of 2.0 mL/min. A single-ramp temperature program was used with an initial temperature of 60 °C, maintained for 1 min, then ramped at 60 °C/min to a temperature of 320 °C maintained for 1 min. This amounted to a total chromatographic time of 6.33 min. The MSD transfer line temperature was set at 280 °C and that of the quadrupole at 150 °C, while the temperature of the source was 230 °C. A solvent delay time of 1 min was set before the source was turned on. All mass spectra were recorded at 70 eV in the electron ionisation (EI) mode. Quantitation was performed in the selected ion-monitoring (SIM) mode. Data collection, integration and signal-to-noise ratio (*S/N*) analysis were performed with Agilent ChemStation software.

2.4. Selected ion monitoring

The following characteristic qualifier ions for the ethyl pentafluorobenzoate were monitored: 212 m/z and 167 m/z, and for the corresponding deuterated internal standard were 213 m/z and 167 m/z. The quantifier ions for the ethyl pentafluorobenzoate and corresponding deuterated internal standard were 240 m/z and 245 m/z respectively.[18,19]

2.5. Blood specimens

Blank whole blood was collected from healthy volunteers in evacuated tubes (Vacutainers) containing heparin anticoagulant in accordance with ethical standards after written informed consent was obtained (Ethical Clearance Number: EC150618-013), and was used as blank matrix throughout the study after verifying that it was negative for ethanol. The individual specimens were refrigerated at 4 ± 3 °C for up to 3 days and pooled before further use. The glucose concentrations of the blood specimens were not determined; however, blood was drawn from young, healthy volunteers and as such can be assumed to be within normal blood glucose limits, namely 72–140 mg/dL.[20]

2.6. Response model and statistical procedures

Response models were calculated with non-weighted linear regression on seven calibration points. The calibration solutions were matrix matched by spiking blood with ethanol standard solutions. The blood ethanol concentrations of the calibration solutions were 0.1001, 0.5005, 2.002, 3.000, 4.004, 5.495 and 7.000 g/L. Internal quality control solutions were also matrix-matched and prepared in the same way as the calibration sample solutions with concentrations of 0.20, 0.50 and 3.00 g/L ethanol in blood. A reagent blank solution was also included. The ethanol concentration result for a specimen was calculated as the mean of duplicate analyses, provided that the duplicates did not differ by more than the precision limits obtained from the expanded measurement uncertainty. The final concentration result was corrected for bias making use of the bias correction plot obtained in Section 2.2 above.

The expanded measurement uncertainty was calculated using internal quality control data at three concentration levels. All concentration values were the mean of two replicates, and were obtained from routine analysis of internal quality control specimens at three concentration levels, which were analysed after every tenth sample in the instrumental sequence. A hybrid of top-down and bottom-up approaches was followed, based on the methodology of Gullberg[21] while including aspects from the SAC-SINGLAS Technical Guide on Measurement Uncertainty in Medical Testing.[22] The overall MU was calculated as the combined contributions of the ethanol certified reference material (CRM), pipettes, bias, and imprecision, and is reported as the expanded MU at both 95% and 99% confidence.

2.7. Storage studies

2.7.1. Sterile ageing study

Two sets of blood specimens were prepared at each of the two ethanol legal limit concentrations (0.20 and 0.50 g/L). One set at each concentration was stored at 4 ± 3 °C, while the remaining two sets were stored at 22 ± 6 °C for 29 weeks. Evacuated collection tubes with sodium fluoride and potassium oxalate preservatives were used to store the blood specimens (7 mL) and a newly opened specimen of each of the sets was analysed once a week in duplicate.

2.7.2. Candida albicans inoculation ageing study

Two sets of blood specimens were spiked at each of the ethanol legal limit concentrations (0.20 and 0.50 g/L) (Fig. 1A), and split into two sets of evacuated tubes with and without NaF (Fig. 1B). Five subsets of specimens were prepared from each of the four resulting sets by inoculating aseptically at different concentrations of *Candida albicans* (1×10^6 cells/mL, 5×10^5 cells/mL, 1×10^4 cells/mL, 5×10^3 cells/mL and 5×10^1 cells/mL) (Fig. 1C). Finally, half of each subset was stored at 4 ± 3 °C, while the remaining half at 22 ± 6 °C for up to nine weeks (Fig. 1D). A newly opened specimen of each of the sets was analysed in duplicate each day for the first 11 days, and then once a week.

2.8. Sample inoculation

A suspension of *C. albicans* comparable to the 0.5 McFarland standard (1.5×10^8 cells/mL) was prepared in PBS, and its optical density confirmed with a bioMérieux Densichek 110 V densitometer (bioMérieux Inc., Durham, NC, USA). (Stock 1).

Stock 1 was then used to prepare a solution of 1.5×10^6 cells/mL (Stock 2), and Stock 2 was further diluted to prepare a solution of 1.5×10^4 cells/ml (Stock 3).

Following this, Stock 1 was used to prepare working solutions at 3.6×10^7 cells/mL (WS 5) and 1.8×10^7 cells/mL (WS 4), Stock 2 to prepare working solutions at 3.6×10^5 cells/mL (WS 3) and 1.8×10^5 cells/mL (WS 2), and Stock 3 to prepare a working solution at 1.8×10^3 cells/mL (WS 1).

Finally, the five subsets of specimens described in Fig. 1C were prepared by adding 200 μL of WS 1–5 to the four sets of tubes seen in Fig. 1B.

2.9. Colony quantification

One hundred microliter of each blood specimen was smeared onto two C-plates, and the plates incubated at 30 °C for 24 h in an aerobic

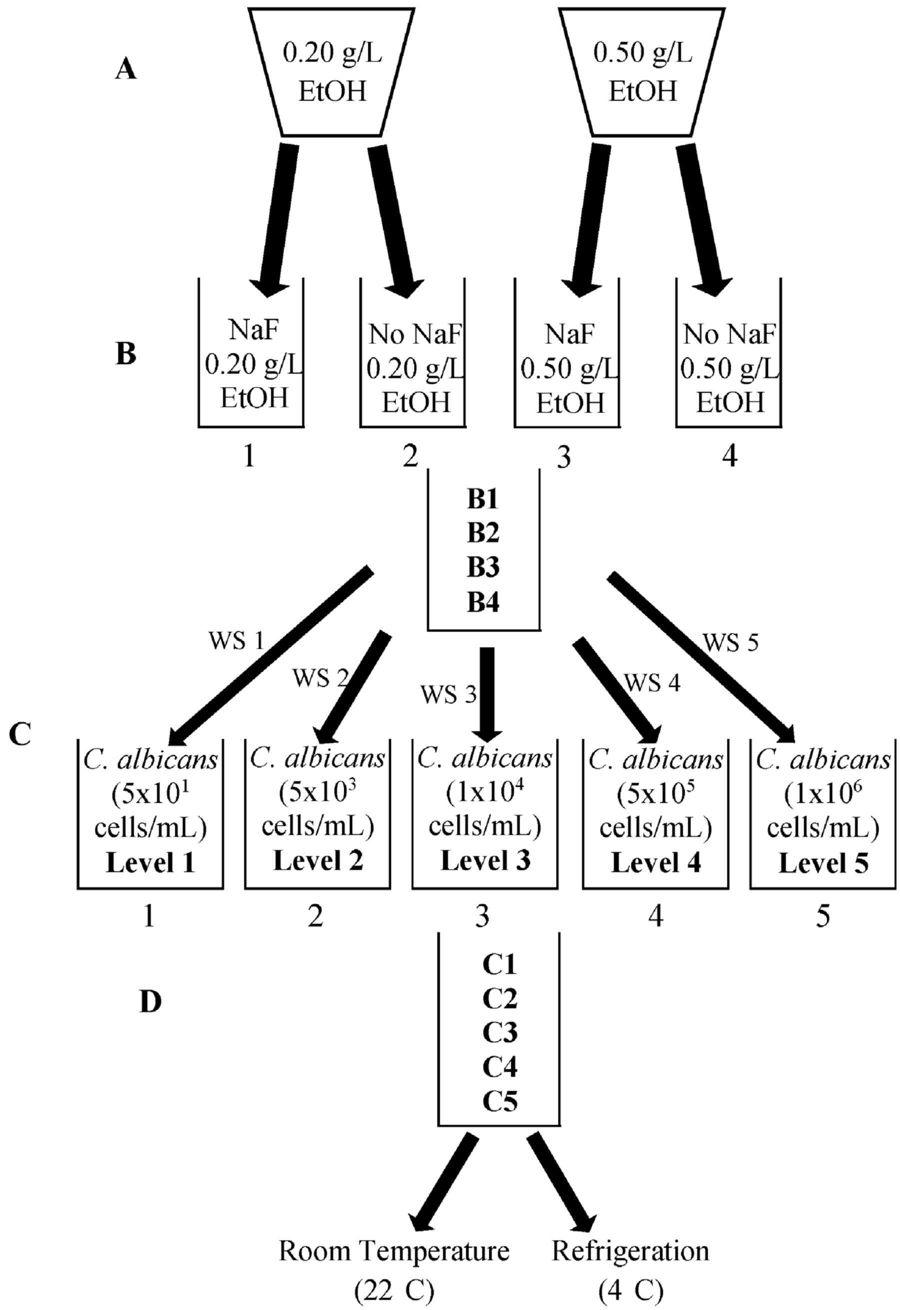

Fig. 1. Preparation of inoculated blood specimens.

incubator (IDS80, manufactured by FMH for PROLAB, Canada). The resulting colonies on each plate were then counted, and the mean (n = 2) for each specimen obtained.

After week 5, specimens stored at room temperature (22 °C) without NaF were diluted 1000 times before being smeared onto the C-plates.

3. Results and discussion

3.1. Precision limits and prosecution concentration levels

The standard measurement uncertainty ($u_{X_0'}$) for the isotope dilution GC-MS method was found during method validation to be 0.028 and 0.053 g/L at the two legal limits 0.20 and 0.50 g/L respectively on a 99% confidence level.

The two-tail precision limits, R, for duplicate test results were calculated by[23]:

$$R = 2.576 \times \sqrt{2} \times u_{X_0'} = 3.643 u_{X_0'} \quad (1)$$

Allowing for a one-tail guard band on a 99% confidence level the prosecution concentration levels for both legal limits were calculated by[21,24,25]:

$$X_{Procecution} = X_{legal\ lim} + 2.576 u_{X_0'} \quad (2)$$

resulting in 0.27 and 0.64 g/L for the 0.20 and 0.50 g/L legal limits respectively. Prosecution can only take place if the reported ethanol concentration is above the upper confidence limit of the analytical method at the cut-off concentration. As such, for the purposes of this study, the ethanol concentration of a stored specimen can only be considered to be significantly increased provided it is above the applicable prosecution concentration level.

3.2. Storage study

With 65% of road incidents in South Africa being alcohol related[26] additional, reliable knowledge of the reliability of blood ethanol concentration test results of stored ante-mortem blood specimens would give valuable guidance for updating and creating laws regarding drunk driving offences.

It is of importance to gauge changes in analyte concentration over time against the expanded measurement uncertainty of a method. Conclusions based on a single test result may be incorrect, since random variation is inevitable for test methods with sufficient sensitivity. There are many literature reports claiming changes in ethanol concentration can occur during specimen storage – with just as many alleging an increase[5,6] in ethanol concentration as those professing a decrease.[27–30] It should, however, be noted that the majority of these studies do not make use of ante-mortem blood but rather post-mortem blood or blood obtained from blood banks which contains additional glucose.

The increase in ethanol concentration during storage is currently used quite effectively as a defence for elevated blood alcohol test results in South Africa.[31] However, statistical significance testing should be applied when assessing the significance of any possible change in ethanol concentration due to storage conditions, such as time, temperature and the presence or absence of NaF. The influence of temperature on blood alcohol concentrations was studied by storing ante-mortem blood specimens spiked with ethanol – sterile and inoculated with *C. albicans* – at 4 °C and 22 °C. The blood specimens contained no additives except the expected NaF and potassium oxalate preservatives where appropriate. The results shown below are all related to the highest concentration of the five *C. albicans* levels; however, the results of the four remaining levels exhibited the same trends.

3.2.1. Refrigeration at 4 °C with NaF

Fig. 2 and Fig. 3 indicate that the ethanol concentrations at both legal limits for the sterile as well as the inoculated specimens stored at 4 °C with NaF remained stable compared to the initial concentrations at a statistical significance of 99%, i.e. there was no significant change in ethanol concentration over the periods of monitoring. The sterile specimens were monitored for 29 weeks and the inoculated specimens until *C. albicans* could no longer be detected – that is, for six and eight weeks for the 0.20 and 0.50 g/L specimens respectively.

3.2.2. Refrigeration at 4 °C without NaF

Despite specimens being inoculated with *C. albicans*, the results in Fig. 4 and Fig. 5 show that the storage of specimens *without NaF but at* 4 °C also results in stable concentrations within the tolerance of the expanded measurement uncertainty at both legal limits. That is, the stability of the ethanol concentration of specimens containing *C. albicans* stored at 4 °C in the absence of NaF is comparable to that of sterile specimens stored at 4 °C in the presence of NaF. The inoculated specimens were monitored for nine weeks, by which time the *C. albicans* was significantly diminished.

In summary, the refrigeration experiment illustrated that blood ethanol concentrations for sterile specimens, spiked at both legal limits, remained stable and within the tolerance of the expanded measurement uncertainty at 4 °C. Additionally, the presence of *C. albicans* had no significant effect on the blood alcohol concentrations at 4 °C up to the point where no colonies of *C. albicans* could be further detected. It was assumed that *C. albicans* could not effect ethanol concentration beyond this point. From the refrigeration experiment it can be concluded that regardless of the presence or absence of NaF in samples – sterile or otherwise – storing specimens at 4 °C was sufficient to stabilise blood alcohol concentrations.

3.2.3. Room temperature at 22 °C with NaF

The ethanol concentrations for the specimens initially spiked at 0.20 and 0.50 g/L and stored at 22 °C with NaF are shown in Fig. 6 and Figure 7 respectively. The inoculated specimens were monitored for six and eight weeks, after which no *C. albicans* could be detected.

The ethanol concentrations at both legal limits for the sterile specimens stored at 22 °C with NaF indicate that there was a significant decrease from the initial concentrations at a 99% confidence level from week 12 onwards. This decrease conforms to the hypothesis of non-enzymatic oxidation of ethanol in blood with haemoglobin as the oxidant.[10–12]

The inoculated specimens for the 0.20 g/L ethanol level showed a significant decrease within two weeks, which seemed to take place at a more rapid rate than that of the sterile specimens. This suggests an additional contribution to the decline of the blood ethanol concentration, which may be attributed to *C. albicans* utilising ethanol as substrate for growth.[4,10–12]

The 0.50 g/L inoculated specimens did not show a significant decrease for up to nine weeks. Closer inspection of the results in Fig. 7 revealed a slight downward trend in the ethanol concentration during the first two weeks, with a decrease in concentration similar to that in the corresponding 0.2 g/L room temperature experiment (Fig. 6). The decrease was not significant however.

3.2.4. Room temperature at 22 °C with no NaF

The results for the room temperature experiment without NaF are summarized in Fig. 8 and Fig. 9.

The inoculated specimens at the 0.20 g/L ethanol level showed the ethanol to be completely depleted within the first two days. The

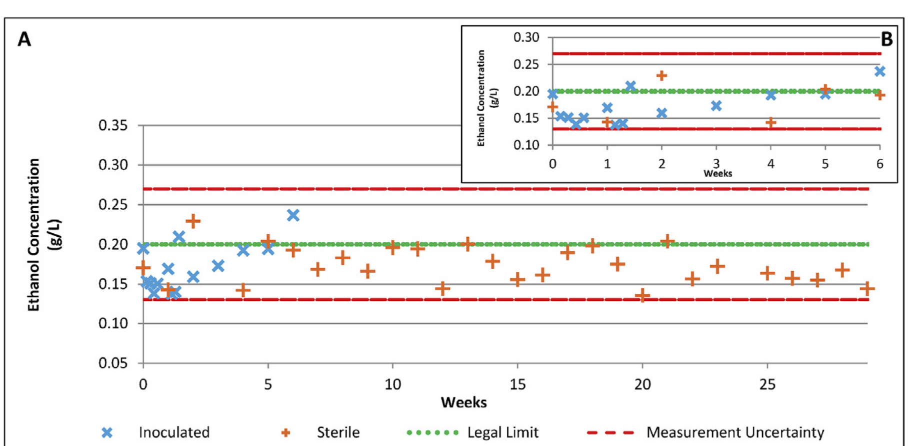

Fig. 2. A: Ethanol concentrations for sterile (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens initially spiked at 0.20 g/L ethanol and stored at 4 °C in the presence of NaF, with the corresponding 99% confidence interval. **B (inlaid)**: Expanded scale of A from week 0 to week 6.

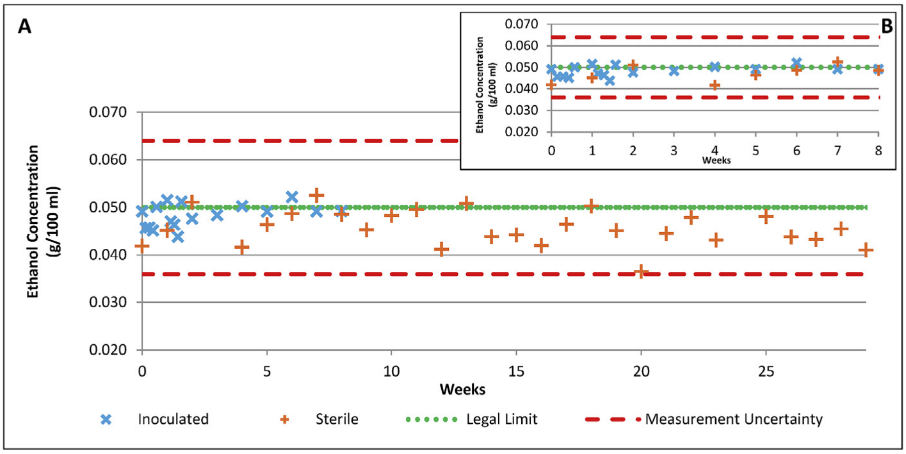

Fig. 3. A: Ethanol concentrations for sterile (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens initially spiked at 0.50 g/L ethanol and stored at 4 °C in the presence of NaF, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 8.

inoculated specimens at the 0.50 g/L ethanol level displayed the same rapid decrease, with the ethanol being almost completely depleted within the first eleven days (Fig. 9). However, thereafter the ethanol concentration increased once more to approximately 0.4 g/L. It was later found that these specimens were possibly contaminated with a *Bacillus* species, and the unexpected increase in ethanol concentration can be attributed to this.[32] Bacillus is an aerobic spore forming bacteria and is found in the environment.[33] It is to be noted that although the presence of the *Bacillus* spp. in the specimens caused an increase, the ethanol concentration was never elevated above the prosecution level of 0.64 g/L, even remaining well below the initial spiked concentration of 0.50 g/L. After termination of the nine week study, high levels of *C. albicans* were still detected in these specimens stored at 22 °C in the absence of NaF.

The results displayed in Figs. 8 and 9 stand in stark contrast to those illustrated in Figs. 6 and 7 where the ethanol decreases steadily for two weeks before levelling off. It is thus clear that, despite the optimum temperature conditions for microbial growth, the presence of NaF has a stabilizing effect on the ethanol concentration. This is due to the NaF inhibiting the anaerobic glycolytic pathway of fermentation, while concurrently impeding its ability to metabolise and break down ethanol.[4,10,34,35] This depletion of ethanol witnessed in Figs. 8 and 9 supports the hypothesis that the *C albicans* uses ethanol as substrate.

In summary, the room temperature experiment illustrated that the storage of blood specimens at room temperature (22 °C) causes significant decreases in blood ethanol concentrations at both legal limits over time, regardless of the presence or absence of NaF. This decrease in ethanol concentration is exacerbated by the presence of *C. albicans*, and the presence of *C. albicans* had a more significant effect on the decrease in ethanol concentration at the lower legal limit of 0.20 g/L as compared to the 0.50 g/L limit. This is potentially explained by the contamination of blood tubes during the preparation process with some other micro-organism, such as *Bacillus* spp., which could have contributed to the blood alcohol concentration at this level.

The temperature at which the specimens are stored has a significant effect on the stability of blood ethanol concentrations.[11] A low temperature (4 °C) keeps microbial growth under control and, with the combined effect of fluoride inhibition, stabilises the ethanol concentrations to remain within the tolerance of the expanded measurement uncertainty. The corresponding experiments at room temperature (22 °C) showed a significant decline in ethanol concentrations at the lower legal limit, even in the presence of fluoride. The initial decrease in ethanol concentration in the 0.50 g/L specimen conforms to the hypothesis that *C. albicans* uses ethanol as a substrate; however, this was followed by an increase in ethanol concentration, possibly due to the presence of an ethanol-generating microbe due to contamination during initial sample preparation. It is thus vital to work aseptically when preparing specimens for such a study.

There was no indication of an increase in blood ethanol concentration beyond the starting concentration levels. It was shown that the blood ethanol concentrations were stabilised by a lowered temperature in combination with fluoride preventing the alcohol

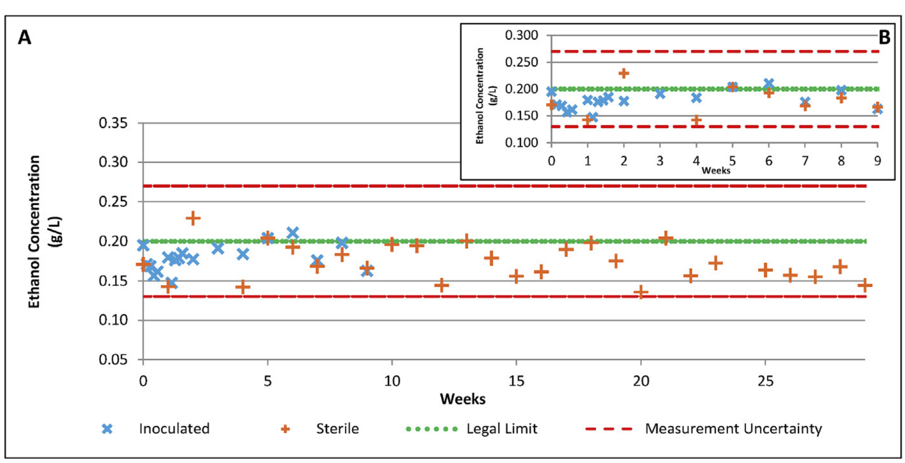

Fig. 4. A: Ethanol concentrations for sterile specimens with NaF (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens without NaF initially spiked at 0.20 g/L ethanol and stored at 4 °C, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 9.

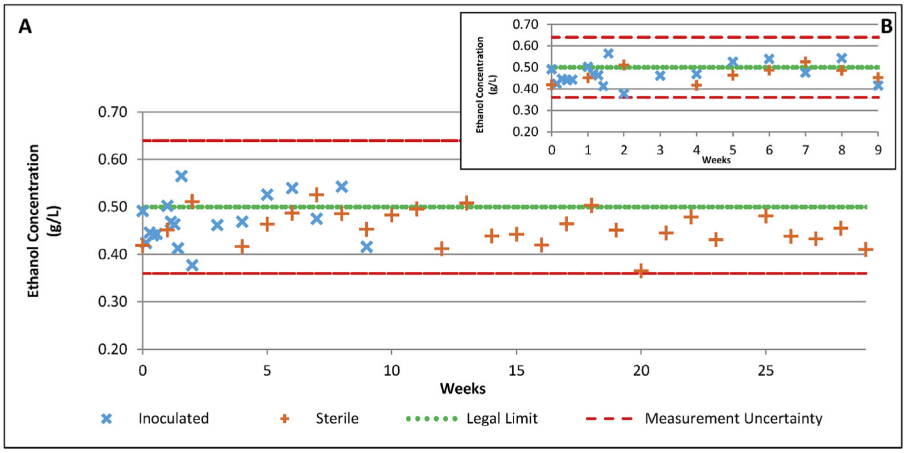

Fig. 5. A: Ethanol concentrations for sterile specimens with NaF (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens without NaF initially spiked at 0.50 g/L ethanol and stored at 4 °C, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 9.

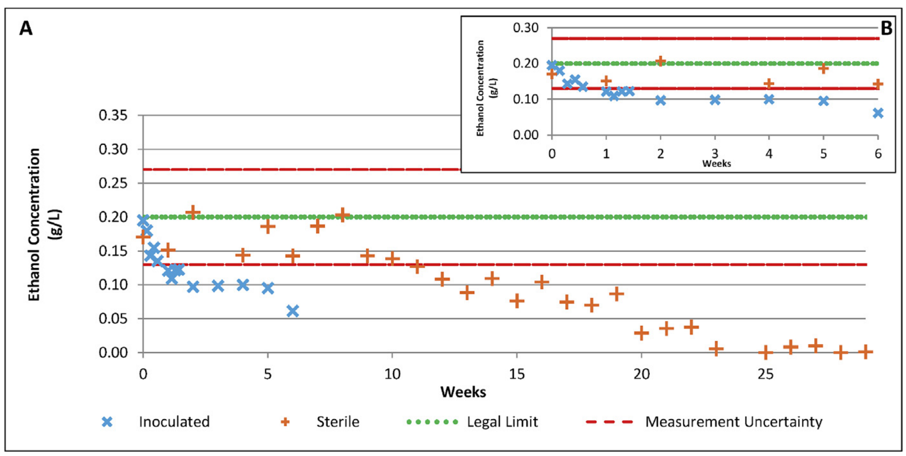

Fig. 6. A: Ethanol concentrations for sterile (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens initially spiked at 0.20 g/L ethanol and stored at 22 °C in the presence of NaF, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 6.

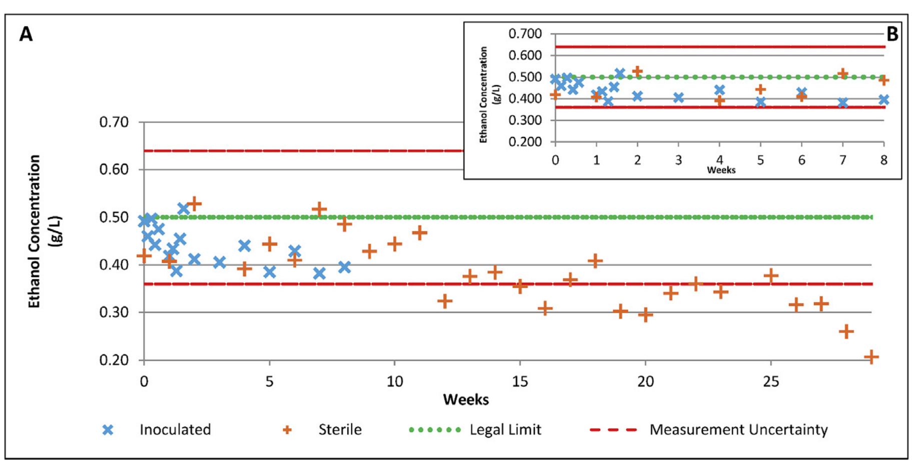

Fig. 7. A: Ethanol concentrations for sterile (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens initially spiked at 0.50 g/L ethanol and stored at 22 °C in the presence of NaF, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of A from week 0 to week 8.

concentrations from decreasing significantly.

It is important to note, however, that since glucose concentrations were not monitored throughout, it is possible that the glucose concentration decreased in the inoculated blood specimens and the only remaining substrate for the *C. albicans* to use for growth was the ethanol. This may potentially explain the decreases in ethanol concentrations observed in the inoculated specimens. Literature studies claiming ethanol increase make use of blood obtained from blood banks[5] which has an additional 400 mg/dL glucose as compared to the ante-mortem blood used in this study. Yajima et al.[7] reported no ethanol increase unless additional glucose was added to the blood used. This would seem to indicate that an increase in ethanol due to microbial

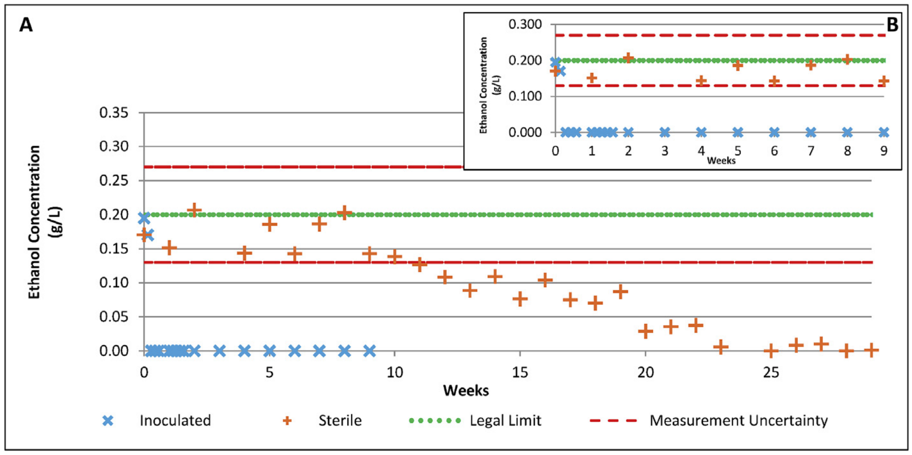

Fig. 8. **A**: Ethanol concentrations for sterile specimens with NaF (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens without NaF initially spiked at 0.20 g/L ethanol and stored at 22 °C, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 9.

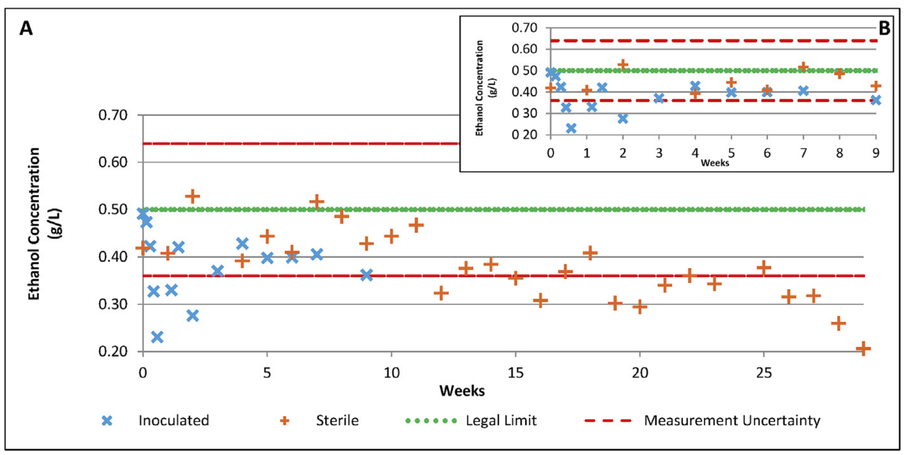

Fig. 9. **A**: Ethanol concentrations for sterile specimens with NaF (plusses) and inoculated (1×10^6 cells/mL, crosses) specimens without NaF initially spiked at 0.50 g/L ethanol and stored at 22 °C, with the corresponding 99% confidence interval. **B (inlaid):** Expanded scale of **A** from week 0 to week 9.

contamination in blood with normal blood glucose levels would be unlikely.

Should this work be repeated, glucose levels should be monitored throughout in order to gain a better understanding of the relationship between ethanol concentration changes and glucose levels.

4. Conclusions

The importance of assessing possible changes in blood alcohol concentrations against the backdrop of the expanded measurement uncertainty is illustrated and is shown to be critical in making reliable assessments in order to draw scientifically correct conclusions about changes in ethanol concentrations. It was crucial that the measurement uncertainty be calculated based on the specific analytical method and under the specific analytical conditions for the laboratory in which the study took place.

Despite the relatively large precision values of the method, significant changes and clear trends in ethanol concentrations were still observed over time in the ageing study.

In general, there was no indication of a significant increase in blood alcohol concentration at either legal limit, whether the specimens were stored under refrigeration or at room temperature, or in the presence or absence of NaF. The ethanol concentrations of the specimens not stored under refrigeration in the presence of NaF typically showed a decreasing trend, except in the case of the 0.50 g/L room temperature specimens without sodium fluoride, which exhibited a drastic decrease followed by an increase to approximately 0.40 g/L.

It was noted that the rate of decrease in ethanol concentration was more dependent on the temperature than on the presence or absence of fluoride, although the presence of NaF did have a stabilizing effect on the ethanol concentration. Hence it is recommended that specimens for blood alcohol analysis be stored under refrigeration at or below 4 °C, in the presence of at least 1% NaF to ensure the stability of the ethanol concentration for up to 29 weeks.

Conflicts of interest

None declared.

Declarations of interest

None.

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